



#5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	<i>Application Number</i>	09/931,267
	<i>Filing Date</i>	August 16, 2001
	<i>First Named Inventor</i>	Clinton C.S. CHAPPLE et al.
	<i>Group Art Unit</i>	1645
	<i>Examiner Name</i>	To Be Assigned
	<i>Attorney Docket Number</i>	N1422-005
<i>Title of the Invention:</i> Gene Encoding P-Coumarate 3-Hydroxylase (C3H) and Methods of Use		

PRELIMINARY AMENDMENT

Hon. Commissioner for Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

In response to the Notice to File Missing Parts of Nonprovisional Application, mailed October 3, 2001, please amend the above-identified application as follows:

Please replace paragraphs numbered [0024], [0026], [0028], [0029], [0032], [0162], [0201] and [0202] of the specification as shown in the attached amended pages.

Marked up copies of the original text of the specification portions are attached to this amendment. Material inserted is indicated by underlining (_____) and material deleted is indicated by brackets [].

IN THE SPECIFICATION

Please replace paragraphs numbered [0024], [0026], [0028], [0029], [0032], [0162], [0201] and [0202] of the specification as follows:

Please replace the paragraph numbered [0024] as follows:

[0024] Figures 1A-C show HPLC analysis of soluble secondary metabolites produced by wild-type and *ref8* plants. (A) Compounds found in wild-type and *ref8* leaves were extracted with methanol and analyzed by HPLC. The elution of UV-absorbing compounds was monitored at 320 nm. (B) Hydroxycinnamic acids released from their ester conjugates by saponification (1M NaOH, 16h, room temperature) of the methanolic extract of (A). pCt, *trans-p*-coumaric acid; pC_c, *cis-p*-coumaric acid; St, *trans*-sinapic acid; S_c, *cis*-sinapic acid. (C) The same analyses as (A) performed on wild-type and *ref8* seed extracts. Sinmal, sinapoylmalate; Singlc, sinapoylglucose; Sincho, sinapoylcholine.

Please replace the paragraph numbered [0026] as follows:

[0026] Figure 3 shows analysis of phenylpropanoid synthesis in a standard, wild-type, and *ref8* leaves using radiotracer feeding technology. Radiotracer feeding and metabolite extraction was performed as in Figure 2 except that extracts were dried and methanolized by incubation in methanolic HCL (80°C, 1h). Products were extracted into diethyl ether and methylcaffeate was purified by semi-preparative silica gel TLC (solvent 1, benzene/dioxane/acetic acid 90:10:1; solvent 2, toluene/acetic acid/water 2:1 sat.), followed by reverse phase HPLC. The continuous line represents the UV-absorption of the HPLC column eluate. The histogram represents radioactivity associated with each fraction. Caf, caffeic acid; pC_{coum}, *p*-coumaric acid; MeCaf, methylcaffeate; MeC_{coum}, methyl *p*-coumarate.

Please replace the paragraph numbered [0028] as follows:

[0028] Figure 5 shows nitrobenzene oxidation products of rachis lignin for wild-type, *ref8* and *fah-1* as analyzed by HPLC. IS, internal standard (3-ethoxy-4-hydroxybenzaldehyde); Bad, *p*-hydroxybenzaldehyde; Sad, syringaldehyde; Van, vanillin, VanA, vanillic acid.

Please replace the paragraph numbered [0029] as follows:

[0029] Figures 6A-C show expression of active C3H in yeast. Yeast carrying the void YeDP60 vector or the YeDP60-C3H vector were cultured and induced as described previously (Humphreys, et al., 1999) in media supplemented with *p*-coumaric acid. At the end of the incubation period the medium was extracted with ethylacetate, and analyzed by HPLC (Fig. 6A). Spectra of the caffeic acid standard, and the co-chromatographing novel peak found in the medium of the yeast carrying YeDP60-C3H are shown in Figs. 6B and C.

Please replace the paragraph numbered [0032] as follows:

[0032] Figures 9A-C are duplicative of Figures 6A-C and Figures 9D-F show results of replicate experiments described in Figure 6A-C when analyzed by GC-MS.

Please replace the paragraph numbered [0162] as follows:

[0162] For cell wall preparation, *Arabidopsis* rachis (stem) tissue was ground to a fine powder and extracted with neutral phosphate buffer, 80% ethanol and acetone (Meyer et al., 1998). Cell wall esterified phenolics were released by saponification with 1M NaOH for 16h at room temperature, and hydrolysis products were extracted in ethyl acetate and separated by reverse phase HPLC using detection at 320 or 275 nm. To measure lignin content, cell wall samples saponified as described above were analyzed using the TGA method (Campbell and Ellis, 1992), or the microscale Klason method (Kaar et al., 1991). Lignin monomer composition of saponified cell walls was determined by nitrobenzene oxidation (Meyer et al., 1998) the DFRC method (Lu and Ralph, 1997) modified as previously described (Franke et al., 2000), and by Py-GC-MS (Bocchini et al., 1997) (Fig. 5). NBO reactions were first extracted with dichloromethane to remove nitrobenzene, after which reaction products were extracted into diethyl ether and analyzed by HPLC. DFRC products were quantified by comparison to authentic standards using GC-MS and selective ion monitoring.

[2021] Finally, WAT11 yeast carrying the control vector and the YeDP60-*REF8* vector were assayed for C3H polypeptide activity *in vivo* by adding *p*-coumarate directly to the medium of galactose-induced yeast cultures. This method has previously been used to demonstrate the activity of C4H and F5H heterologously expressed in yeast (Pierrel et al., 1994; Humphreys et al., 1999), and exploits the ability of simple hydroxycinnamic acids to readily cross yeast membranes. When *p*-coumarate was added to the medium of control yeast, it was the predominant UV-absorbent substance present in the medium after several hours of incubation. In contrast, when a parallel experiment was performed with yeast harboring the YeDP60-*REF8* expression vector, a novel peak was found whose retention time and UV-spectrum matched precisely those of caffeic acid (Fig. 6). Replicate experiments analyzed by GC-MS with selective ion monitoring permitted unequivocal identification of the C3H reaction product. (Fig. 9) In the context of the phenotypic characterization of the *ref8* mutant (Franke et al., submitted), these data provide definitive proof that *CYP98A3* encodes C3H polypeptide, and that C3H polypeptide is a P450.

Please replace the paragraph numbered [0202] as follows:

[0202] Although the *in vivo* assays of yeast carrying the YeDP60-REF8 vector demonstrated that C3H polypeptide is capable of hydroxylating *p*-coumarate, it was not possible to use this approach to determine kinetic constants for the enzyme, nor to use this system to assay the activity of C3H toward substrates that cannot readily cross the yeast plasma membrane. To experimentally address these issues, C3H-containing microsomes were prepared for use in *in vitro* assays of enzymatic activity. Consistent with the *in vivo* results, incubation of C3H in the presence of *p*-coumarate *in vitro* resulted in the production of caffeic acid, although this activity was so low that it precluded detailed kinetic analysis. In addition to *p*-coumarate, several other compounds have been suggested to be substrates for the 3-hydroxylase(s) of phenylpropanoid metabolism (Heller and Kühnl, 1985; Kühnl et al., 1987; Kneusel et al., 1989; Tanaka and Kojima, 1991). Because the assays using *p*-coumarate suggested that it may not be the optimal substrate for C3H, we assayed the activity of the enzyme against an array of other possible substrates. In these experiments, no activity of C3H polypeptide toward *p*-coumaroyl CoA, *p*-hydroxycinnamyl alcohol, and 1-*O*-*p*-coumaroyl--D-glucose was detected. Levels of activity comparable to those seen with *p*-coumarate were seen when *p*-coumaraldehyde was used as a substrate. In contrast, much higher levels of activity were seen when *p*-coumaroyl methyl ester was used as a substrate (Fig. 7a), although the apparent K_m for this substrate (2.5 ± 0.1 mM) was still higher than those of other phenylpropanoid pathway P450s. No activity was seen when cinnamate, caffeate or ferulate was used as a substrate for C3H.

REMARKS

The Notice to File Missing Parts mailed October 3, 2001, states that Figures 3A-C and 5A-C described in the Specification appear to be missing. Applicant notes that the Description of the Figures section of the Specification filed in this matter included typographical errors indicating that Figures 3 and 5 are numbered 3A-C and 5A-C when they are actually Figures 3 and 5, without separate letters. This typographical error is corrected at paragraphs **[0026]**, **[0028]** and **[0162]** of the Specification by amendment herein.

Applicant notes that additional amendments are made herein in order to correct several other typographical errors in the Specification associated with description of and reference to the Figures. Specifically, at paragraph [0024], definitions are provided for the abbreviations included in Fig. 1B as filed. Support for this amendment is found in the Specification, for example in paragraph [0182].

Paragraph [0028] is further amended to correct the description of Figure 5. Support for this amendment is found in the Specification, for example at paragraphs [0162] and [0192].

Paragraph [0029] is amended to clarify that Figure 6 (as filed) includes three parts, 6A-C.

Paragraph [0032] is amended to correctly describe Figures 9A-F. Support for this amendment is found in the specification, for example in paragraphs [0201].


Paragraph [0202] is amended to delete two erroneous references to Fig. 9.

These amendments do not add new matter and their entry is requested.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached pages are captioned **"Version with markings to show changes made."**

A Response to Notice to File Missing Parts with the missing Declarations and Powers of Attorney are being filed concurrently with this Preliminary Amendment.

Applicant believes there are no fees due; however, if any fees are due, please charge Deposit Account 02-2135 in the name of Rothwell, Figg, Ernst & Manbeck.

SIGNATURE OF APPLICANT, ATTORNEY OR AGENT REQUIRED					
NAME AND REG. NUMBER	Nancy T. Morris, Reg. No. 42,017				
SIGNATURE		DATE	November 28, 2001	DEPOSIT ACCOUNT USER ID	

Attachments: Marked-Up Copies of Amended Specification

Amended Specification: Version with markings to show changes made

IN THE SPECIFICATION

Please replace the paragraph numbered [0024] as follows:

[0024] Figures 1A-C show HPLC analysis of soluble secondary metabolites produced by wild-type and *ref8* plants. (A) Compounds found in wild-type and *ref8* leaves were extracted with methanol and analyzed by HPLC. The elution of UV-absorbing compounds was monitored at 320 nm. (B) Hydroxycinnamic acids released from their ester conjugates by saponification (1M NaOH, 16h, room temperature) of the methanolic extract of (A). pCt, *trans-p*-coumaric acid; pCc, *cis-p*-coumaric acid; St, *trans*-sinapic acid; Sc, *cis*-sinapic acid. (C) The same analyses as (A) performed on wild-type and *ref8* seed extracts. Sinmal, sinapoylmalate; Singlc, sinapoylglucose; Sincho, sinapoylcholine.

Please replace the paragraph numbered [0026] as follows:

[0026] Figure[s] 3[A-C] shows analysis of phenylpropanoid synthesis in [A)] a standard, [B)] wild-type, and [C)] *ref8* leaves using radiotracer feeding technology. Radiotracer feeding and metabolite extraction was performed as in Figure 2 except that extracts were dried and methanolized by incubation in methanolic HCL (80°C, 1h). Products were extracted into diethyl ether and methylcaffeate was purified by semi-preparative silica gel TLC (solvent 1, benzene/dioxane/acetic acid 90:10:1; solvent 2, toluene/acetic acid/water 2:1 sat.), followed by reverse phase HPLC. The continuous line represents the UV-absorption of the HPLC column eluate. The histogram represents radioactivity associated with each fraction. Caf, caffeic acid; *p*Coum, *p*-coumaric acid; MeCaf, methylcaffeate; MeCoum, methyl *p*-coumarate.

Please replace the paragraph numbered [0028] as follows:

[0028] Figure[s] 5[A-C] shows nitrobenzene oxidation products of rachis lignin for [analysis of lignin monomer composition of] wild-type, [and] *ref8* and *fah-1* as[. (A) Nitrobenzene oxidation products] analyzed by HPLC[,]. IS, internal standard (3-ethoxy-4-hydroxybenzaldehyde); Bad [*p*OHBen], *p*-hydroxybenzaldehyde; Sad [Syr], syringaldehyde; Van, vanillin, VanA, vanillic acid. [(B) DFRC products analyzed by GC-MS using selective ion monitoring. CAD, coniferyl alcohol diacetate; *p*CAD, *p*-coumaryl alcohol diacetate; IS, internal standard (4,4'-ethylidene-bisphenol), SAD, sinapyl alcohol diacetate. (C) Lignin analysis by Py-CG-MS. G₁-G₁₂, guaiacyl lignin pyrolysis products; H₁-H₅, *p*-hydroxyphenyl

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lignin pyrolysis products; IS, internal standard (1,3,5-tri-*t*-butylbenzene); S₁-S₃ syringyl lignin pyrolysis products.]

Please replace the paragraph numbered [0029] as follows:

[0029] Figures 6A-C show[s] expression of active C3H in yeast. Yeast carrying the void YeDP60 vector or the YeDP60-C3H vector were cultured and induced as described previously (Humphreys, et al., 1999) in media supplemented with *p*-coumaric acid. At the end of the incubation period the medium was extracted with ethylacetate, and analyzed by HPLC (Fig. 6A). Spectra of the caffeic acid standard, and the co-chromatographing novel peak found in the medium of the yeast carrying YeDP60-C3H are shown [at right] in Figs. 6B and C.

Please replace the paragraph numbered [0032] as follows:

[0032] Figures 9A-C are duplicative of Figures 6A-C and Figures 9D-F show[s] results of replicate experiments described in Figure 6A-C when analyzed by GC-MS [kinetic analysis of C3H-catalyzed substrate 3-hydroxylation. (A) Comparison of relative activity of C3H using *p*-coumarate, *p*-coumaraldehyde, *p*-coumaryl alcohol, and *p*-coumarate methyl ester at a concentration of 1 mM. (B) Lineweaver-Burke plot of *p*-coumaroyl methyl ester 3-hydroxylation. For both graphs, error bars represent one standard deviation for triplicate assays].

Please replace the paragraph numbered [0162] as follows:

[0162] For cell wall preparation, Arabidopsis rachis (stem) tissue was ground to a fine powder and extracted with neutral phosphate buffer, 80% ethanol and acetone (Meyer et al., 1998). Cell wall esterified phenolics were released by saponification with 1M NaOH for 16h at room temperature, and hydrolysis products were extracted in ethyl acetate and separated by reverse phase HPLC using detection at 320 or 275 nm. To measure lignin content, cell wall samples saponified as described above were analyzed using the TGA method (Campbell and Ellis, 1992), or the microscale Klason method (Kaar et al., 1991). Lignin monomer composition of saponified cell walls was determined by nitrobenzene oxidation (Meyer et al., 1998) the DFRC method (Lu and Ralph, 1997) modified as previously described (Franke et al., 2000), and by Py-GC-MS (Bocchini et al., 1997) (Fig. 5). NBO reactions were first

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extracted with dichloromethane to remove nitrobenzene, after which reaction products were extracted into diethyl ether and analyzed by HPLC. DFRC products were quantified by comparison to authentic standards using GC-MS and selective ion monitoring.

Please replace the paragraph numbered [0201] as follows:

[0201] Finally, WAT11 yeast carrying the control vector and the YeDP60-*REF8* vector were assayed for C3H polypeptide activity *in vivo* by adding *p*-coumarate directly to the medium of galactose-induced yeast cultures. This method has previously been used to demonstrate the activity of C4H and F5H heterologously expressed in yeast (Pierrel et al., 1994; Humphreys et al., 1999), and exploits the ability of simple hydroxycinnamic acids to readily cross yeast membranes. When *p*-coumarate was added to the medium of control yeast, it was the predominant UV-absorbent substance present in the medium after several hours of incubation. In contrast, when a parallel experiment was performed with yeast harboring the YeDP60-*REF8* expression vector, a novel peak was found whose retention time and UV-spectrum matched precisely those of caffeic acid (Fig. 6). Replicate experiments analyzed by GC-MS with selective ion monitoring permitted unequivocal identification of the C3H reaction product. (Fig. 9) In the context of the phenotypic characterization of the *ref8* mutant (Franke et al., submitted), these data provide definitive proof that *CYP98A3* encodes C3H polypeptide, and that C3H polypeptide is a P450.

Please replace the paragraph numbered [0202] as follows:

[0202] Although the *in vivo* assays of yeast carrying the YeDP60-*REF8* vector demonstrated that C3H polypeptide is capable of hydroxylating *p*-coumarate, it was not possible to use this approach to determine kinetic constants for the enzyme, nor to use this system to assay the activity of C3H toward substrates that cannot readily cross the yeast plasma membrane. To experimentally address these issues, C3H-containing microsomes were prepared for use in *in vitro* assays of enzymatic activity. Consistent with the *in vivo* results, incubation of C3H in the presence of *p*-coumarate *in vitro* resulted in the production of caffeic acid [(Fig. 9)], although this activity was so low that it precluded detailed kinetic analysis. In addition to *p*-coumarate, several other compounds have been suggested to be substrates for the 3-hydroxylase(s) of phenylpropanoid metabolism (Heller and Kühnl, 1985; Kühnl et al., 1987; Kneusel et al., 1989; Tanaka and Kojima, 1991). Because the assays using

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p-coumarate suggested that it may not be the optimal substrate for C3H, we assayed the activity of the enzyme against an array of other possible substrates. In these experiments, no activity of C3H polypeptide toward *p*-coumaroyl CoA, *p*-hydroxycinnamyl alcohol, and 1-*O*-*p*-coumaroyl--D-glucose was detected. Levels of activity comparable to those seen with *p*-coumarate were seen when *p*-coumaraldehyde was used as a substrate. In contrast, much higher levels of activity were seen when *p*-coumaroyl methyl ester was used as a substrate (Fig. 7a), although the apparent K_m for this substrate (2.5 ± 0.1 mM[; Fig. 9]) was still higher than those of other phenylpropanoid pathway P450s. No activity was seen when cinnamate, caffeate or ferulate was used as a substrate for C3H.

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